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Identification of *Yersinia* at the species and subspecies levels is challenging

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Abstract

The genus *Yersinia* currently includes 18 species, of which *Y. enterocolitica* and *Y. pseudotuberculosis* are enteropathogenic. The identification of *Y. enterocolitica* in particular is very demanding, because it consists of a group of very heterogeneous bacteria, including pathogenic and non-pathogenic strains. The aim of the review is to provide recent information on the characteristics and identification of *Yersinia* spp. and sources of enteropathogenic *Yersinia* spp. Identification of *Yersinia* spp. is still mainly based on biochemical tests and serotyping, but molecular methods have increasingly also been used. Sequencing the whole genome enables more accurate identification of enteropathogenic *Yersinia* spp. Pathogenic *Y. enterocolitica* strains of different bioserotypes have newly been identified from various animal sources. Moreover, the virulence gene *ail* has been detected in non-pathogenic *Yersinia* strains, especially from wild animals. Correct identification of pathogenic *Yersinia* strains is essential in assessing the health risk for humans and animals.

Keywords *Yersinia* · taxonomy · characteristics · identification · subtyping · sources

Introduction

The genus *Yersinia* is large and diverse, currently consisting of 18 species (1·). The enteropathogenic *Yersinia* spp., *Y. enterocolitica* and *Y. pseudotuberculosis* are important foodborne pathogens, mostly causing self-limiting enteritis in humans and an asymptomatic infection in animals (1·,2). Human and animal cases are mainly sporadic and outbreaks are rare (3·). Human yersiniosis is usually due to *Y. enterocolitica*, and is still the third most commonly reported enteritis in Europe, thus the correct identification of these enteropathogenic *Yersinia* is essential for making correct diagnoses, and for preventing new infections (4). However, identification of *Yersinia* to the species and subspecies levels can be very demanding, especially the identification of pathogenic *Y. enterocolitica* (5·). *Y. enterocolitica* is a very heterogeneous species including six biotypes and phylogenetic groups varying from non-pathogenic to highly pathogenic strains (6,7). *Y. enterocolitica* and *Y. pseudotuberculosis* are widely found in various animal species. However, pathogenic *Y. enterocolitca* strains have mostly been isolated from pigs at slaughter (2,3·).

Taxonomy of the genus *Yersinia*

The taxonomy of genus *Yersinia*, which belongs to the family Enterobacteriaceae, has experienced wide changes over the years (8-10). Presently it comprises 18 species (*Y. aldovae, Y. aleksiciae, Y. bercovieri, Y. entomophaga, Y. enterocolitica, Y. frederiksenii, Y. intermedia, Y. kristensenii, Y. massiliensis, Y. mollaretii, Y. nurmii, Y. pekkanenii. Y. pestis, Y. pseudotuberculosis, Y. rohdei, Y. ruckeri, Y. similis* and *Y. wautersii*) (1·,3·,11). Enteropathogenic *Y. enterocolitica* and *Y. pseudotuberculosis* together with plague-associated *Y. pestis* are the three species virulent for humans and animals, *Y. ruckeri* is a fish pathogen, *Y. entomophaga* is an insect pathogen and the rest are more or less environmental species rarely associated with human or animal diseases (5·). *Y.*

wautersii is the latest species that formed a clearly distinct non-pathogenic population of strains in the *Y. pseudotuberculosis* complex group; however, Neubauer and Sprague (12) claimed that *Y. wautersii* should continue to be classified as the *Y. pseudotuberculosis* complex and not as a separate species. *Y. nurmii* and *Y. entomophaga* have recently been reported to be very closely related (13). *Y. enterocolitica* species is a very heterogeneous group of bacteria including both pathogenic and non-pathogenic strains (5·). Currently, *Y. enterocolitica* is divided into two subspecies based on the 16S rRNA gene sequence: subsp. *enterocolitica* including high-pathogenic strains and subsp. *palearctica* including low-pathogenic and non-pathogenic strains. However, it appears that European non-pathogenic *Y. enterocolitica* strains form at least one own subspecies (14,15). Moreover, Sihvonen et al. (16) presented two different phylogenetic clusters of *Y. enterocolitica* 1A strains based on seven housekeeping genes (Table 1). Using whole-genome sequencing, *Y. enterocolitica* species has newly been divided into six phylogenetic groups (5·).

Characteristics of *Yersinia* spp.

Members of *Yersinia* spp. are gram negative, facultative anaerobic rod-shaped bacterium (8). The size of complete *Yersinia* genomes, except *Y. ruckeri*, which is clearly smaller, range from 4.0 to 4.9 Mb with G+C contents ranging from 47 to 49% (www.ncbi.nlm.nih.gov/genome/). *Yersinia* bacteria are psychrotrophic and thus capable of growing at low temperatures below 6°C (2). Cold enrichment at 4°C for one to three weeks has been widely used to isolate *Yersinia* from clinical, food and environmental samples (2,17,18). *Yersinia* spp. tolerate freezing for a longer time, but are heat-sensitive. They also tolerate alkaline conditions better than many other bacteria, and thus alkali treatment with potassium hydroxide (KOH) has been used to reduce the level of other bacteria during *Yersinia* isolation (17,18). Most *Yersinia* spp., including the two enteropathogenic species *Y. enterocolitica* and *Y. pseudotuberculosis*, are urease-positive (10). Urea testing is widely used for

confirmation of suspected enteropathogenic *Yersinia* colonies (17-19). *Yersinia* strains are typically resistant to beta-lactam antibiotics due to beta-lactamase genes located in the chromosome (20). However, *Y. pseudotuberculosis* strains are usually susceptible to tested antimicrobials while *Y. enterocolitica* strains are more often resistant (19). Resistance to streptomycin, sulphonamide and tetracycline among *Y. enterocolitica* strains has recently been reported in Italy, Spain and Iran (19,21,22). Resistance to chloramphenicol, ciprofloxacin, nalidixic acid and sulfamethoxazole-trimethoprim has also been shown (19-22).

The three human pathogenic *Yersinia* spp. (*Y. enterocolitica*, *Y. pseudotuberculosis* and *Y. pestis)* carry the approximately 70-kb virulence plasmid (pYV), which is essential for their survival and ability to multiply in different lymphoid tissues of the host (1,10,23). All correctly identified *Y. pseudotuberculosis* strains are considered pathogenic whereas *Y. enterocolitica* sp. also includes non-pathogenic strains not carrying the pYV (1,3). The high pathogenicity island HPI is only found in the high-pathogenic *Y. enterocolitica* strains and frequently in *Y. pseudotuberculosis* O:1 and O:3 strains (2,10,24). Some *Y. pseudotuberculosis* strains more commonly found in the Far East can also synthesise a superantigen toxin YPM (*Y. pseudotuberculosis* -derived mitogen) (25). YPM plays an important role in systemic infections, especially in the disease called Far East scarlet fever (FESLF) primarily observed in Japan and Russia (25,26). Based on the virulence in the mouse model, *Y. enterocolitica* can be divided into three groups consisting of highly pathogenic, weakly pathogenic and non-pathogenic strains (10). Non-pathogenic *Y. enterocolitica* strains typically lack the chromosomal virulence genes *ail* and *yst*A but carry the *yst*B gene (7,21,27). Interestingly, the *ail* gene has also quite recently been detected more frequently in *Y. enterocolitica* biotype 1A strains, especially in 1A strains from wildlife, but recently also from sheep and lettuce (12,28-34). Joutsen et al. (29) reported newly *ail*-positive *Y. kristensenii* strains isolated from voles.

The two human pathogens, *Y. enterocolitica* and *Y. pseudotuberculosis*, are transmitted faecal-orally and colonise the intestinal tract, especially the Peyer's patches in the terminal ileum (1,10,23,35). The clinical picture varies depending on the patient's age and immune system, and the pathogenicity of the strain. The symptoms of *Y. enterocolitica* and *Y. pseudotuberculosis* infections can be quite similar (2). The most common symptoms are diarrhoea and fever, which occur especially in young children (22,36). Both bacteria may produce terminal ileitis and mesenteric lymphadenitis, which are sometimes accompanied with secondary infections, such as reactive arthritis and erythema nodosum, occurring more commonly in adolescents and adults (22,23,35). Human enteropathogenic yersiniosis occurs mostly sporadically, and outbreaks, especially due to *Y. enterocolitica*, are rare (2,4,37,38). *Y. pseudotuberculosis* outbreaks have more often been reported in Finland, Japan and Russia (2,25). Outbreaks due to *Y. pseudotuberculosis* infection linked to contaminated raw milk and produce have recently been reported in Finland and New Zealand, respectively (39,40). Worldwide, the most common types associated with human infections are *Y. enterocolitica* bioserotypes 2/O:9 and 4/O:3, and *Y. pseudotuberculosis* serotypes O:1 and O:3 (41). The high-pathogenic *Y. enterocolitica* O:8, which typically belongs to biotype 1B, has newly been identified in sporadic human yersiniosis with varying symptoms in Japan (38).

Identification of *Yersinia* spp.

Several selective agar plates have been designed for isolation and identification of *Yersinia* spp. from different sources (17,42). CIN (cefsulodin-irgasan-novobiocin) agar plates are still mostly used for *Yersinia* isolation (7,17,18,43). However, this medium is not optimal for all pathogenic strains such as *Y. enterocolitica* biotype 3 strains and some *Y. pseudotuberculosis* strains. Furthermore, pathogenic strains sometimes grow as very small colonies missing the typical red centre on the CIN plates (18,42). Chromogenic-based agar plates have become popular in recent

years for isolation and identification of *Y. enterocolitica* belonging to pathogenic biotypes (42). CHROMagar *Yersinia* (CAY), which is currently the only commercial chromogenic medium, is not suitable for *Y. pseudotuberculosis* isolation (2,43). Tan et al. (42) recently designed a modified CIN agar plate that provided a better discrimination of *Yersinia* colonies from other bacteria than traditional CIN agar. A stereomicroscope has been used to aid in the identification of characteristic *Yersinia* colonies on selective agar plates for further confirmation (17,18).

Yersinia spp. are still mostly identified by commercial biochemical identification systems like API20E, API50CH, Microgen™ GN-ID and Biolog Microbial ID (3,19,44-46). However, biochemical reactions do not guarantee reliable identification at species level, as only minor differences often exist between species (1,2,46,47). Especially, non-pathogenic *Yersinia* spp. are difficult to differentiate from *Y. enterocolitica*, and *Y. similis* and *Y. wautersii* similarly from *Y. pseudotuberculosis* (12,47). Joutsen et al. (29) recently reported that differentiating between sucrose-negative *Y. enterocolitica* and *Y. kristensenii* and identification of *Y. pseudotuberculosis* is impossible when only using API20E. Furthermore, identification of bioserotype 5/O:(1,2,)3 strains isolated from sheep is very challenging (30).

Identification of *Yersinia* spp. by matrix-assisted laser desorption/ionisation time-of-flight (MALDI-TOF) mass spectrophotometry has emerged as a rapid and accurate technology that provides protein profiles for the identification of *Yersinia* at the species and subspecies levels (33,48-52). Rizzardi et al. (49) designed a protocol that was able to correctly identify the common pathogenic bioserotypes 2/O:9, 2/O:5,27 and 4/O:3. However, biochemical methods are still needed to distinguish non-pathogenic biotype 1A strains from highly pathogenic 1B strains. Differentiation between *Y. pseudotuberculosis*, *Y. pestis* and *Y. similis* is also challenging due to their tight genetic

147 relationship (48). Thus, comprehensive databases are needed, and additional confirmatory testing in
148 some cases as well.
149
150 Methods based on sequencing are more accurate for identifying *Yersinia* spp. than methods based
151 on phenotypic characteristics (2). 16SrRNA gene sequencing is widely used for investigation of
152 taxonomic relationships including species identification; however, in many bacterial species
153 including *Yersinia* spp., the 16SrRNA gene sequences show high similarity and lack enough
154 variation to differentiate species or subspecies (46,47). Murros et al. (15) recently showed that the
155 discriminatory power of 16SrRNA sequencing is too low to discriminate non-pathogenic *Y.*
156 *enterocolitica* 1A strains from the pathogenic *Y. enterocolitica* strain belonging to biotypes 2-5.
157 Multilocus sequence analysis (MLSA), which has a higher resolution than 16SrRNA gene
158 sequencing, has widely been used in taxonomic studies to determine phylogenetic relationships of
159 *Yersinia* strains (11,15,47,53,54). The four housekeeping genes *gln*A, *gyr*B, *hsp*60 and *rec*A based
160 on Kotetishvili et al. (55) have been used in most taxonomic studies of *Yersinia* spp. (11,15,53,54).
161 In multilocus sequence typing (MLST), sequences of approximately 500 bp of at least seven
162 housekeeping genes have been indexed to identify the sequence type of the studied isolate (56).
163 Seven housekeeping genes according to Hall et al. (5) have mainly been used to characterise *Y.*
164 *enterocolitica* isolates (34,57) and seven housekeeping genes according to Laukkanen-Ninios et al.
165 (58) for *Y. pseudotuberculosis* isolates (26,39,59) (Table 1). Duan et al. (59) developed an MLST
166 scheme based on the housekeeping genes according to Laukkanen et al. (58) for the three
167 pathogenic *Yersinia* spp. and Hall et al. (5) developed a pan-*Yersinia* MLST scheme for accurate
168 and reproducible identification of *Yersinia* isolates. The sequence data are freely available and the
169 allelic profiles of isolates can be compared to those in central databases
170 (http://enterobase.warwick.ac.uk/species/index/yersinia, https://pubmlst.org/yersinia/).
171

Serotyping *Y. enterocolitica* and *Y. pseudotuberculosis* is still an approach for diagnostics to identify these two pathogens and to assess their potential pathogenicity, especially for *Y. enterocolitica* (8,10). Classical serotyping performed by slide agglutination using commercial monovalent and polyvalent sera is very simple and cost-effective, however, several drawbacks exist: interpretation is occasionally challenging and both false-positive and false-negative results may occur. Especially serotypes associated with pathogenicity, such as O:3, O:8 and O:9, may appear among non-pathogenic *Y. enterocolitica* strains and other non-pathogenic *Yersinia* spp. (27,60). Furthermore, a cross-reaction between serotype O:9 and *Brucella* occurs (61,62). Recently Garzetti et al. (27) designed a PCR-based typing scheme for identifying *Y. enterocolitica* serotypes O:3, O:5,27, O:8 and O:9, which are the most common serotypes associated with human and animal diseases. In a recent study, Bozcal et al. (51) used PCR-based O-antigen genotyping and serotype-specific bacteriophages for identification of *Y. enterocolitica* serotypes O:3, O:5,27 and O:9.

Biotyping based on a series of biochemical reactions is still widely used for *Y. enterocolitica* because biotypes correlate with the potential pathogenicity of this species (3,7,8). Strains of biotypes 1B and 2 to 5, which are associated with yersiniosis, carry the virulence plasmis (pYV) and chromosomal virulence genes *ail* and *yst*A (27). Biotype 1A strains, which are considered non-pathogenic, lack the pYV and usually carry *yst*B (60). However, biotyping can be very demanding due to untypical biochemical reactions (5). Recently, six phylogroups (PG) of *Y. enterocolitica* strains have been proposed using whole genome sequencing (5,14). PG1 consists of the non-pathogenic biotype 1A strains, PG2 of highly pathogenic biotype 1B strains and PG3-PG6 include low-pathogenic strains due to their lethality in a mouse model (5). Alenizi et al. (63) have recently demonstrated that PG1 strains exhibit high levels of virulence in an insect infection model, and thus should no longer be described as non-pathogenic strains. There is also a close association between bioserotype and the source of the strain (6,7).

PCR methods permit rapid identification of pathogenic *Yersinia* strains with high specificity (27). For pathogenicity, pYV-encoded targets, such as *yad*A and *vir*F, and chromosomal target genes *ail* and *yst*A are widely used (19,20,36,51,64). Species-specific regions of virulence genes have also been used for correct identification of enteropathogenic *Yersinia* (29). The new ISO method uses *ail* PCR to identify pathogenic *Y. enterocolitica* on selective agar plates before further confirmation (18). However, *ail* has frequently been detected in *Y. enterocolitica* biotype 1A strains isolated from wildlife, but also recently in lettuce, and this gene should therefore not be used alone in PCR detection when searching for pathogenic strains (28,29,31).

Enteropathogenic *Yersinia* spp. in animal sources

Culturing, PCR detection and immunoassays have been applied to identify pathogenic *Y. enterocolitica* and *Y. pseudotuberculosis* in clinical, food and environmental samples, and serological analyses have widely been used for indirect detection of enteropathogenic *Yersinia* in asymptomatic animals, especially in fattening pigs but also recently in wild boars (2,19,41,45,65-68). Serological tests have also proven valuable, especially when enteropathogenic *Yersinia* has not been identified from the faeces of a patient with sequelae, such as arthritis, after gastroenteritis (69).

Domestic pigs and wild boar have shown to be important reservoirs for pathogenic *Y. enterocolitica* and *Y. pseudotuberclosis* (3,32,65,66,70). Bioserotype 4/O:3 of *Y. enterocolitica* is still the dominant type identified in tonsils, faeces, mandibular lymph nodes and on the carcasses of slaughter pigs (19,36,71-73) (Table 2). Surprisingly, this type has also newly been identified in pigs and humans in West Africa (36). Bioserotype 4/O:3 strains have also been found in pork, which appears to be an important infection source for human infections (3,74). In Asia, *Y. enterocolitica*

O:3 strains are commonly identified in pigs and dogs, however, these strains mostly belong to biotype 3 (32). Interestingly, bioserotype 3/O:3 has also newly been identified for the first time in pet hamsters in Japan (75). Bozcal et al. (51) recently found bioserotypes 2/O:5,27 and 2/O:9 in pig manure in Turkey, indicating that pigs may also be a reservoir for these types. *Y. pseudotuberculosis* O:1 and O:3 are the most common serotypes identified in human infections in Europe, and the same types have newly been reported by Bonardi et al. (19) in pig tonsils in Italy. In wild boar, a great variety of serotypes have been identified. *Y. enterocolitica* bioserotypes 2/O:5,29, 2/O:9 and 4/O:3, and *Y. pseudotuberculosis* O:1 and O:2 have been found in Switzerland (76). Recently, Bancerz-Kisiel et al. (28) identified *Y. enterocolitica* 4/O:3 in wild boars in Poland and Arrausi-Subiza et al. (65) *Y. pseudotuberculosis* O:1 in northern Spain.

Enteropathogenic *Yersinia* have been found in domestic ruminants only sporadically (3) (Table 2). Recently, Yang et al. (77) reported quite high prevalence (15%) of enteropathogenic *Yersinia* including both *Y. enterocolitica* and *Y pseudotuberculosis* in sheep tonsils in Austria using PCR detection. *Y. enterocolitica* bioserotype 2/O:9, which is the second most common type after bioserotype 4/O:3 in human infections, has recently been identified in sheep in Finland (30). In the same study, bioserotype 5/O:(1,2,)3, was found for the first time in Finnish sheep. This rare bioserotype has previously been associated with wild hares in Europe (3). In Ireland and Poland, *Y. enterocolitica* O:9 has frequently been identified in cattle, especially in animals showing false-positive serological activity to *Brucella* (61,62). Interestingly, bioserotypes 1B/O:8 and 4/O:3 have been found in raw cow milk in Iran, indicating that cows may also be a reservoir for these types (21). During a *Y. pseudotuberculosis* O:1 outbreak in Finland due to contaminated raw milk, the same serotype was found in both milk and cattle faeces from the same farm (40). *Y. pseudotuberculosis* is a rare finding in cows, but serotypes O:1-O:3 have sporadically been identified in ruminants in France (3).

Wildlife is an important reservoir of enteropathogenic *Yersinia*, especially *Y. pseudotuberculosis* (24). In Italy, *Y. pseudotuberculosis* O:1 was the most common type identified in wild boar, hare and deer (24). The same type has also newly been identified in brown rats in Belgium (33). *Y. pseudotuberculosis* O:2, which has more frequently been identified in animals than in humans, was recently reported in Finnish shrews (3,29). Pathogenic *Y. enterocolitica* strains have more rarely been identified in wild animals except for wild boars. However, highly pathogenic bioserotype 1B/O:8 has recently been identified in wild rodents in Japan (78) and weakly pathogenic bioserotypes 2/O:5,27 and 3/O:1,2,3 in brown rats in Belgium (33). Non-pathogenic *Y. enterocolitica* 1A has proven a common finding in wild animals (29,79).

Y. pseudotuberculosis outbreaks have frequently been reported in captive animals, especially in non-human primates (80), but also in captive birds and rodents (44,81-83). Recently, serotype O:1 was found in dead Amazonian parrots in Italy (81). Several serotypes have been recently identified in captive animals in Japan: serotypes O:1 and O:4 in squirrel monkeys, serotypes O:2 and O:4 in dead toucans, O:1 in a dead squirrel and O:4 in dead meerkats (80,82,84) (Table 2). In outbreaks with high mortality, *Y. pseudotuberculosis* has shown to be quite easily identified from the spleen and liver using selective CIN agar plates, and biochemical and serological testing (81,84).

Conclusions

Yersiniosis due to *Y. enterocolitica* and *Y. pseudotuberculosis* infections is still the third most frequently reported zoonotic enteric disease in Europe (4). Correct identification of pathogenic *Yersinia* strains is essential to determine the relevance of the isolated strains in human and animal infections. Biochemical tests are still widely used for identifying *Yersinia* spp. and for subtyping

(biotyping) *Y. enterocolitica* strains, although untypical reactions are common and interpretation is demanding. Potential pathogenicity of the strains is mostly confirmed by PCR based on essential virulence genes. In the future, whole genome sequencing with *in silico* analysis of the data will probably be the best strategy to identify and subtype *Yersinia* spp.

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Table 1

Housekeeping genes used for multilocus sequence analysis (MLSA) and typing (MLST) of *Yersinia* spp.

Genes	MLSA	MLST applied mostly for		References
		Yersinia enterocolitica	*Yersinia pseudotuberculosis*	
aarF		x		(5,34,57)
adk			x	(16,26,39,58,59)
argA			x	(16,26,39,58,59)
aroA			x	(16,26,39,58,59)
dfp		x		(5,34,57)
galR		x		(5,34,57)
glnA	x		x	(11,15,16,26,39,53-55,58,59)
glnS		x		(5,34,57)
gyrB	x			(11,15,16,47,53-55)
hemA		x		(5,34,57)
hsp60	x			(11,15,47,53-55)
recA	x			(11,15,53-55)
rfaE		x		(5,34,57)
rpoB	x			(47)
sodA	x			(47)
speA		x		(5,34,57)
thrA			x	(16,26,39,58,59)
tmk			x	(26,39,58,59)
trpE			x	(16,26,39,58,59)

Table 2

Recently identified pathogenic *Yersinia enterocolitica* (YE) and *Yersinia pseudotuberculosis* (YP) in various animal sources.

Animals	YE	YP	BT/ST	Country	Reference
Captive meerkats		x	O:4	Japan	(84)
Captive monkeys		x	O:1, O:4, O:6	Japan	(80)
Captive squirrels		x	O:1	Japan	(82)
Captive toucans		x	O:2, O:4	Japan	(82)
Cattle	x		O:9	Ireland, Poland	(61,62)
Chickens	x		3/O:3	China	(32)
Dogs	x		3/O:3	China	(32)
Goats	x		3/O:3	China	(32)
Pet hamsters	x		3/O:3	Japan	(75)
Pet parrots		x	O:1	Italy	(81)
Pigs	x		4/O:3	Africa	(36)
	x		2/O:9, 3/O:3	China	(32)
	x		O:3	Croatia	(71)
	x		4/O:3	Finland	(73)
	x		4/O:3	Italy	(19)
		x	O:1, O:3	Italy	(19)
	x		4/O:3	Netherlands	(72)
		x	O:1, O:2	Netherlands	(72)
	x		4/O:3, 2/O5,27, 2/O:9	Turkey	(51)
Sheep	x		2/O:9, 5/O:(1,2,)3	Finland	(30)
		x	O:1	Italy	(24)
Shrews		x	O:2	Finland	(29)
Voles	x		2/O:9	Finland	(29)
Wild boars		x	O:1	Spain	(65)
	x		4/O:3	Poland	(28)
Wild rodents	x		2/O:5,27, 3/O:1,2,3	Belgium	(33)
		x	O:1	Belgium	(33)
	x		1B/O:8	Japan	(78)
	x		2/O:9, 3/O:3	China	(32)